

Analysis of Plant Toxins

1 Introduction

Plants contain a variety of chemicals and compounds, many of which can be toxic. Examples include alkaloids such as gelsemine (*Gelsemium*) and glycosides such as digoxin/digitoxin (*Digitalis*), oleandrin (*Nerium*), and cerberin (*Cerbera*).

2 Scope

Analyses	<input checked="" type="checkbox"/> Screening <input checked="" type="checkbox"/> Confirmation <input type="checkbox"/> Quantitation
Matrices	Whole blood (0.2 mL per extraction).
Analytes	Digoxin, digitoxin, cerberin, oleandrin, gelsemine.
Personnel	This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

3 Principle

Specimens are diluted and adjusted to basic pH through a combination of aqueous buffers and organic solvent. The resulting solution is mixed and centrifuged. The supernatant is applied to a supported liquid extraction (SLE) column. Organic solvents are used to elute the analytes from the column. The eluent is concentrated, reconstituted and filtered. The prepared extract is analyzed by UPLC-HRMS/MS (ultra- performance liquid chromatography-high resolution tandem mass spectrometry). Three acquisition modes are utilized: full scan (FS; 35,000 resolution), selected ion monitoring (SIM; 35,000 resolution), and tandem mass spectrometry (MS²; 17,500 resolution).

4 Procedure

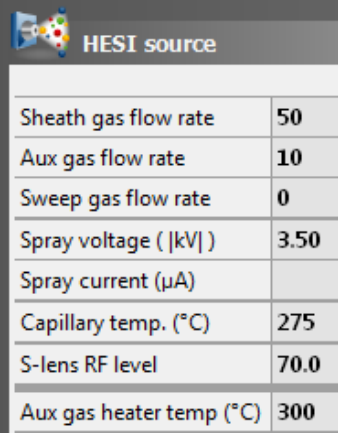
Step	Activity	Material	Reference/Lot
4.1	Materials required per sample: 2 mL Eppendorf tube (1), SLE+ 400 µL cartridge (1), 12 x 75 mm glass tube (1), 0.2 µm centrifugal filter (1), ALS vial (1)		
4.2	Thaw a control set (maintained at -20°C). (0, 1 and 10 ng/mL Controls, 200 µL each; System Suitability Sample (S ³), 10 ng/mL)	Control Lots, S³	[] ₄
4.3	Aliquot 200 µL of each case specimen into a 2 mL Eppendorf tube.		
4.4	Add 100 µL of Sample Buffer to each tube. (0.1 M sodium phosphate, pH 6.8)	Sample Buffer	[]
4.5	Add 50 µL of Internal Standard Solution (ISS)	ISS	[]
4.6	— Add 50 µL of pH Modifier. Cap vial. (scan NH ₄ OH)	pH Modifier	[]
4.7	Vortex at 2000 rpm for 5 minutes at ambient temperature.		
4.8	Centrifuge at 10,000 rpm for 5 minutes at ambient temperature.		
4.9	Load Biotage SLE+ 400 µL cartridges onto positive pressure manifold. Place 12 x 75 mm tubes beneath.	Biotage SLE+ 400 µL	[]
4.10	Apply 300 µL of supernatant to SLE+ cartridge		
4.11	Apply a short pulse of maximum nitrogen pressure to load sample onto cartridge. Wait 5 minutes.		
4.12	Apply 750 µL of Elution Solvent 1 to each cartridge (95:5 dichloromethane:isopropanol). Wait 5 minutes.	Elution Solvent 1	[]
4.13	Apply 750 µL of Elution Solvent 1 to each cartridge. Wait 5 minutes. Apply low nitrogen flow for ~ 30 seconds to elute Elution Solvent 1.		
4.14	Apply 750 µL of Elution Solvent 2 to each cartridge (MTBE). Wait 5 minutes.	Elution Solvent 2	[]
4.15	Apply 750 µL of Elution Solvent 2 to each cartridge. Wait 5 minutes. Apply low nitrogen flow for ~ 30 seconds to elute Elution Solvent 2.		
4.16	Evaporate eluent to dryness at 45°C. Let cool for 5 min.		
4.17	Reconstitute with 100 µL of Reconstitution Solvent to the bottom of the 12 x 75 mm tube. Vortex well.	Reconstitution Solvent	[]
4.18	Transfer 100 µL extract to 0.2 µm centrifugal filter. Centrifuge at 10,000 rpm for 5 minutes.	Costar 0.2 µm filter	[]
4.19	Transfer extract to Waters ALS vial with 250 µL insert. Cap with Waters pre-slit 12 x 32 mm vial cap.		
4.20	Analyze 20 µL of extract using the parameters in Section 5		

5 Instrument Parameters

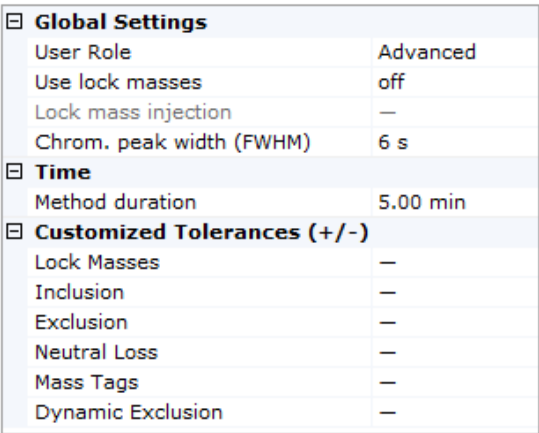
Thermo Fisher Q-Exactive with Waters Acquity I-Class UPLC System

5.1 Mass Spectrometry

5.1.1 Heated Electrospray Ionization, Global Settings and Tune File

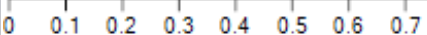


HESI source	
Sheath gas flow rate	50
Aux gas flow rate	10
Sweep gas flow rate	0
Spray voltage (kV)	3.50
Spray current (µA)	
Capillary temp. (°C)	275
S-lens RF level	70.0
Aux gas heater temp (°C)	300




Global Settings	
User Role	Advanced
Use lock masses	off
Lock mass injection	—
Chrom. peak width (FWHM)	6 s
Time	
Method duration	5.00 min
Customized Tolerances (+/-)	
Lock Masses	—
Inclusion	—
Exclusion	—
Neutral Loss	—
Mass Tags	—
Dynamic Exclusion	—

C:\Xcalibur\methods\TOX350.mstune



5.1.2 Inclusion List

Method editor — Inclusion List										
File Edit Help									Done 	
	Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	(N)CE	MSX ID	Comment
▶ 1	323.17540	C20H22N2O2	+ H	1	Positive	1.40	2.00	60		gelsemine
2	798.46343	C41H64O14	+ NH4	1	Positive	2.90	3.10	10		digoxin
3	577.33711	C32H48O9	+ H	1	Positive	3.32	3.62	10		cerberin and oleandrin
4	782.46852	C41H64O13	+ NH4	1	Positive	3.58	3.90	10		digitoxin
* 5										

The start/stop times listed are nominal. Due to normal column aging and variation in mobile phase preparation, small adjustments to the start and stop times may be required based upon the system suitability sample results.

5.1.3 Scan Events

Properties of Full MS — SIM

General	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Full MS — SIM	
Microscans	1
Resolution	35,000
AGC target	5e5
Maximum IT	50 ms
Number of scan ranges	1
Scan range	300 to 840 m/z
Spectrum data type	Profile



Properties of Targeted-SIM

General	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Inclusion	on
SIM	
Microscans	1
Resolution	35,000
AGC target	2e5
Maximum IT	200 ms
MSX count	1
Isolation window	1.0 m/z
Isolation offset	0.0 m/z
Spectrum data type	Profile











Properties of PRM

General	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Default charge state	1
Inclusion	on
MS²	
Microscans	1
Resolution	17,500
AGC target	2e5
Maximum IT	100 ms
Loop count	1
MSX count	1
MSX isochronous ITs	on
Isolation window	1.0 m/z
Isolation offset	0.0 m/z
Fixed first mass	—
(N)CE / stepped (N)CE	nce: 35
Spectrum data type	Profile



5.2 Liquid Chromatograph (LC) Parameters

5.2.1 LC Materials

Component	Description	Reference/Lot
Solvent A1	5mM ammonium formate in water	
Solvent B1	Methanol	
Solvent A2	Methanol:Water 50:50	
Solvent B2	Acetonitrile	
Weak Needle Wash (WNW)	Methanol:Water 10:90	
Strong Needle Wash (SNW)	Methanol:Acetonitrile:Water:Isopropanol 45:40:10:5	
Seal Wash (SW)	Acetonitrile:Water 10:90	
UPLC Column	Waters Acquity UPLC HSS C18 1.8 µm, 2.1 x 100 mm	


5.2.2 Solvent Manager

ACQ-SM ACQ-BSM

Binary Solvent Manager

General | Analog Out | Events

Solvents

A1 5mM ammonium format 


B1 Methanol

Pressure Limits

Low: 0 psi

High: 15000 psi

Seal Wash: ?

2.0 min 

Gradient

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	0.200	50.0	50.0	Initial
2	0.50	0.200	50.0	50.0	6
3	2.50	0.200	5.0	95.0	6
4	3.00	0.200	5.0	95.0	6
5	3.05	0.200	50.0	50.0	6
6	5.00	0.200	50.0	50.0	6
7					

Gradient Start:

☒ At injection

☐ Before injection

☐ After injection

0 uL

5.2.3 Sample Manager

ACQ-SM | ACQ-BSM

Sample Manager

General | Events

Wash Solvents

Weak Wash Name:
10/90 methanol/water

Strong Wash Name:
45/40/10/5 Strong Wash

Weak Wash Volume:
1200 μ L

Strong Wash Volume:
800 μ L

Max Sample Volume: 15.00 μ L

Comment:

Temperature Control

Column: 50.0 $^{\circ}$ C Alarm Band: ☐ \pm 5.0 $^{\circ}$ C

Sample: 14.0 $^{\circ}$ C ☐ \pm 5 $^{\circ}$ C

Loop Offline:
Disable min

☐ Load Ahead

Active Preheater:
Enabled

Advanced...

6 Equipment/Materials/Reagents

6.1 Chemicals and Consumables

Item	Supplier*	Description	Part Number*
Eppendorf Tubes	Eppendorf	Safe-Lock Tubes 2.0mL (polypropylene)	0030 120.094
SLE Cartridge	Biotage	Isolute SLE+, 400 μ L sample volume	820-0055-B-500
Glass Tube	Fisher	Disposable Culture Tube 12x75 mm	14-961-26
Centrifugal Filter	Corning	Costar Spin-X HPLC 0.2 μ m with nylon filter	8169
ALS Vials	Waters	Screw Top Vial, 12x32 mm, PTFE/Silicone pre-slit cap (with 250 μ L insert)	186000307C
Water	Fisher	Optima, LC-MS grade (mobile phase and Reconstitution Solvent)	W6-4

Water	In-house	18 mΩ, deionized	n/a
Methanol	Thermo Scientific	UPLC-MS grade (mobile phase preparation)	A458
Methanol	Fisher	Optima LC-MS grade (sample preparation and solvents)	A4 54-4
Acetonitrile	Fisher	Optima LC-MS grade	A955-5
Isopropanol	Fisher	Optima grade	A4 51
Ammonium formate	Fisher	Optima LC-MS grade	A1 15
Dichloromethane	Fisher	Optima grade	D151-1
MTBE (Elution Solvent 2)	Sigma-Aldrich	Chromasolv, 99.9%	20257
Sodium phosphate, monobasic, monohydrate	Fisher	Certified ACS	S3 69
Sodium phosphate, dibasic, heptahydrate	Fisher	Certified ACS	S3 73
Ammonium hydroxide	Fisher	ACS Plus	A669S
Negative Control Matrix	Cliniqa	Blood	n/a
*use of an equivalent product is allowable			

6.2 Prepared Mixtures and Solvents

Depending upon the batch size, the absolute amounts may be adjusted so long as the ratios of components are maintained.

6.2.1 Sample Buffer (0.1 M sodium phosphate buffer, pH 6.8)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 50 mL
2	Add	40 mL	deionized water
3	Add	656 mg	sodium phosphate, dibasic, heptahydrate
4	Add	352 mg	sodium phosphate, monobasic, monohydrate
5	QS	50 mL	deionized water
6	Mix		
7	Transfer		amber glass
8	Store		refrigerated
	Stability		1 month
	Prepares	50 mL	(500 samples)

6.2.2 pH Modifier (2% ammonium hydroxide)

Step	Action	Amount	Component/Information
1	Acquire	1	eppendorf Tube, 2 mL polypropylene
2	Add	2.0 mL	deionized water
3	Add	41 µL	ammonium hydroxide
4	Mix		
5	Store		in tube
	Stability		1 day
	Prepares	2 mL	(40 samples)

6.2.3 Elution Solvent 1 (95:5 dichloromethane:isopropanol)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 100 mL
2	Add	57 mL	dichloromethane
3	Add	3 mL	isopropanol
4	Mix		
5	Transfer		amber glass
6	Store		ambient
	Stability		1 year
	Prepares	60 mL	(40 samples)

6.2.4 Reconstitution Solvent, Solvent A2 (50:50 methanol:water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 25 mL
2	Add	12.5 mL	water (Optima LC-MS)
3	Add	12.5 mL	methanol (UPLC-MS grade)
4	Mix		
5	Transfer		glass
6	Store		ambient or refrigerated or frozen
	Stability		6 months
	Prepares	25 mL	(250 samples)

6.2.5 Solvent A1 (5mM ammonium formate in water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	250 mL	water (Optima LC-MS)
3	Add	79 mg	ammonium formate (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Store		ambient or refrigerated
	Stability		10 days
	Prepares	250 mL	

6.2.6 Weak Needle Wash (WNW) (10:90 methanol:water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	225 mL	water (Optima LC-MS)
3	Add	25 mL	methanol (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Store		ambient
	Stability		3 months
	Prepares	250 mL	

6.2.7 Strong Needle Wash (SNW) (45:40:10:5 Methanol:Acetonitrile:Water:Isopropanol)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 500 mL
2	Add	225 mL	methanol (Optima LC-MS)
3	Add	200 mL	acetonitrile (Optima LC-MS)
4	Add	50 mL	water (Optima LC-MS)
5	Add	25 mL	isopropanol (Optima)
6	Mix		
7	Transfer		mobile phase bottle, glass
8	Store		ambient
	Stability		1 year
	Prepares	500 mL	

6.2.8 Seal Wash (SW) (10:90 acetonitrile:water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	225 mL	water (Optima LC-MS)
3	Add	25 mL	acetonitrile (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Store		ambient
	Stability		3 months
	Prepares	250 mL	

7 Standards and Controls

7.1 Primary Standards

Analyte	Supplier*	Description	Part Number*
Cerberin	Santa Cruz Biotechnology	1 mg powder	SC-480467
Digoxin	Cerilliant	1.0 mg/mL in methanol	D-029
Digitoxin	Cerilliant	1.0 mg/mL in methanol	D-067
Oleandrin	Phytolab	10 mg powder	89744
Gelsemine	Phytolab	10 mg powder	80457
Digoxin-d3	Cayman Chemicals	1 mg powder	10010657
*Use of an equivalent product is allowable. Store at about -20°C. Stability determined by manufacturer			

7.2 Primary Standards in Methanol from Solid

For the standards in section 7.1 that are in solid form, perform a dilution to yield a 1.0 mg/mL solution in methanol. For example, remove 1.0 mg of the oleandrin primary standard and add 1.0 mL of methanol. Store at about -20°C in amber glass.

7.3 Intermediate Standards (10 µg/mL in methanol)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	2.5 mL	methanol (Optima LC-MS)
3	Add	50 µL	of each 1.0 mg/mL primary standard (excluding digoxin-d3)*
4	QS	5 mL	methanol (Optima LC-MS)
5	Mix		
6	Transfer		amber glass
7	Store		about -20°C
	Stability		2 years
*Make a separate Intermediate Standard containing digoxin-d3 only (internal standard)			

7.4 Working Standard (0.25 µg/mL in methanol)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	2.5 mL	methanol (Optima LC-MS)
3	Add	125 µL	of Intermediate Standard (Section 7.3)
4	QS	5 mL	methanol (Optima LC-MS)
5	Mix		
6	Transfer		amber glass
7	Store		about -20°C
	Stability		2 years

7.5 Controls (0, 1 and 10 ng/mL in matrix)

Prepare controls according to the table below. Mix each bulk control solution for 30 minutes prior to pipetting into Eppendorf centrifuge tubes (0.2 mL portions each). Store at about -20°C. Stable for two years.

Control Level	Working Standard (Section 7.4) µg/mL	Addition Volume µL	Matrix Volume mL	Concentration ng/mL
Negative	0.25	0	5	0
1 ng/mL	0.25	20	5	1
10 ng/mL	0.25	200	5	10

7.6 Internal Standard Solution (80 ng/mL in methanol)(ISS)

Aliquot 40 µL of the digoxin-d3 10 µg/mL solution (from Section 7.3) to a 5 mL glass volumetric flask. QS with methanol (Optima LC-MS). Store at about -20°C in amber glass. Stable for two years.

7.7 System Suitability Sample (S³)(10 ng/mL)

Prepare the S³ portions according to the table below.

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	1.7 mL	methanol (Optima LC-MS)
3	Add	200 µL	of Working Standard (Section 7.4)
4	Add	625 µL	of ISS (Section 7.6)
5	QS	5 mL	water (Optima LC-MS)
6	Mix		
7	Transfer		eppendorf vials in 0.2 mL portions
8	Store		about -20°C along with controls
	Stability		2 years

8 Decision Criteria

In order for a chromatographic peak to be used for identification, the following criteria must be met:

Retention Time	Mass Accuracy	Signal To Noise	Preceding Negative Sample Response
± 5 % of concurrent standard or extracted control	± 5 mmu	≥ 3	≤ 10

8.1 Analyte Specific Decision Criteria

Analyte	Scan Mode	Retention Time†	Adduct / Fragment	m/z
Digoxin	SIM	2.99	M+NH ₄	798.463
	MS ²	2.99	Fragment	651.373
			Fragment	97.065
			Fragment	391.247
	MS ² spectra are concentration dependent. Refer to TOX104.			
		2.99	M+NH ₄	798.463

	Full Scan*		M+H	781.436
	<i>*The inclusion of full scan data is optional. Digoxin undergoes in-source fragmentation, as well as forms multiple adducts.</i>			
Digitoxin	SIM	3.65	M+NH ₄	782.469
	MS ²	3.65	Fragment	635.380
			Fragment	97.065
			Fragment	375.253
	<i>MS² spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	3.65	M+NH ₄	782.469
	<i>*The inclusion of full scan data is optional. Digitoxin forms primarily the ammonium adduct.</i>			
Cerberin	SIM	3.53	M+H	577.337
	MS ²	3.53	Fragment	203.091
			Fragment	171.065
	<i>MS² spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	3.53	M+H	577.337
			M+NH ₄	594.364
	<i>*The inclusion of full scan data is optional. Cerberin forms primarily the protonated adduct as well as an ammonium adduct at a lower abundance.</i>			
Oleandrin	SIM	3.41	M+H	577.337
	MS ²	3.41	Fragment	373.237
			Fragment	433.258
			Fragment	113.060
	<i>MS² spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	3.41	M+H	577.337
		M+NH ₄	594.364	
	<i>*The inclusion of full scan data is optional. Oleandrin forms primarily the protonated adduct as well as an ammonium adduct at a lower abundance.</i>			
Gelsemine	SIM	1.63	M+H	323.175
	MS ²	1.63	Fragment	70.065
			Fragment	236.106
			Fragment	195.067
	<i>MS² spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	1.63	M+H	577.337
	<i>*The inclusion of full scan data is optional. Gelsemine does not form additional adducts.</i>			
Digoxin-d3	Full Scan*	2.98	M+NH ₄	801.482

† The retention times listed are nominal. Due to normal column aging and variation in mobile phase preparation, small adjustments to the start and stop times may be required based upon the system suitability sample results.

8.2 Batch Acceptance

8.2.1 Control Criteria

Target analytes will not be detected in the Negative Control. The S³, 1 and 10 ng/mL Positive Control will have all target analytes identified. Either a positive control or an unextracted standard may be used for mass spec/ion ratios comparisons as needed. For an individual case, the target analytes required may vary.

8.2.2 Internal Standard

The internal standard will be recovered via full scan for each control and unknown sample.

8.2.3 Planned Action on QC Failure

Refer to TOX101 for potential responses to QC failure(s).

9 Limitations

9.1 Limit of Detection (LOD)

Analyte	Matrix	LOD (ng/mL)
Digoxin	Blood	0.5
Digitoxin	Blood	1
Cerberin	Blood	0.1
Oleandrin	Blood	0.1
Gelsemine	Blood	0.1

9.2 Interferences, Isomers, and Interpretation

No interferences identified. Cerberin and oleandrin are isotopomeric isomers. Baseline or near baseline resolution of these two analytes is required to differentiate on the basis of the protonated ion alone. However, the analytes do have different tandem mass spectra. While digoxin (and digitoxin, to a lesser extent) are available as highly purified preparations for medical use, other plant toxins are often present in unprocessed or less purified forms. Potential poisonings from these types of scenarios may generate multiple analytes and metabolites that may be similar in structure and mass spectra to validated analytes. A combination of full scan, SIM, and MS² analyses may be used to investigate potential additional analytes of interest.

10 Sampling

Not applicable.

11 Calculations

Not applicable

12 Measurement Uncertainty

Not applicable.

13 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

14 References

Bylda, Caroline, et al. "Rapid Quantification of Digitoxin and Its Metabolites Using Differential Ion Mobility Spectrometry-Tandem Mass Spectrometry." *Analytical Chemistry*, U.S. National Library of Medicine, 17 Feb. 2015, www.ncbi.nlm.nih.gov/pubmed/25588102.

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0	07/09/2019	New document

Approval

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Quality Manager:

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